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Detection of *Thelohania solenopsae* (Microsporidia: Thelohaniidae) in *Solenopsis invicta* (Hymenoptera: Formicidae) by multiplex PCR

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Abstract

Oligonucleotide primer pairs were designed to unique areas of the small subunit (16S) rRNA gene of *Thelohania solenopsae* and a region of the *Gp-9* gene of *Solenopsis invicta*. Multiplex PCR resulted in sensitive and specific detection of *T. solenopsae* infection of *S. invicta*. The *T. solenopsae*-specific primer pair only amplified DNA from *T. solenopsae* and *T. solenopsae*-infected *S. invicta*. This primer pair did not produce any amplification products from DNA preparations from uninfected *S. invicta*, seven additional species of microsporidia (including *Vairimorpha invictae*), or *Mattesia* spp. The *Gp-9*-specific primers recognized and amplified DNA from *Solenopsis xyloni*, *Solenopsis richteri*, *Solenopsis geminata*, the *invicta/richteri* hybrid, and monogyne and polygyne *S. invicta*, but not from *T. solenopsae*, and, as such, served as a positive control verifying successful DNA preparation. Multiplex PCR detected *T. solenopsae* in worker fire ants infected with as few as 5000 spores. Furthermore, multiplex PCR detected *T. solenopsae* in all developmental stages of *S. invicta.* However, detection could be made more sensitive by using only the *T. solenopsae*-specific primer pair; ants infected with as few as 10 spores were able to be discerned. Multiplex PCR detection of *T. solenopsae* offers the advantages of a positive control, a single PCR amplification, detection of all developmental stages, and increased sensitivity and specificity compared with microscopy.

Keywords: *Thelohania solenopsae*; *Solenopsis invicta*; Multiplex PCR; Diagnostic test

1. Introduction

After its introduction in the 1930s, the red imported fire ant, *Solensopsis invicta*, established itself as the unrivaled pest ant species in the southeastern United States. Early attempts at eradication eventually yielded to quarantine and the pursuit of sustainable control methods in hope of establishing an ecological balance (Williams et al., 2001). In areas where *S. invicta* is indigenous, the infestation rate is a fraction of that observed in the United States which has been attributed to the lack of natural enemies in introduced areas (Porter et al., 1992).

Thelohania solenopsae, a microsporidian parasite of *S. invicta* and *Solensopsis richteri* (Briano et al., 1995a; Knell et al., 1977), has been found recently in the United States (Williams et al., 1998). This obligate intracellular protist significantly reduced fire ant populations in Argentina (Briano et al., 1995b) and caused declines in egg production, queen weight, and worker and queen survivorship in *S. invicta* colonies in the United States (Oi and Williams, 2002; Williams et al., 1998). Hence, *T. solenopsae* appears to be a promising biological control candidate for *S. invicta*. Currently, diagnosis of *T. solenopsae* infection is limited to microscopic examination. Unfortunately, the inability to easily discern vegetative states of the organism has hampered epidemiologic studies, elucidation of the life cycle, and identification of possible intermediate hosts. Such studies will be crucial for the successful utilization of this pathogen as a biological control agent.

Several methods have been developed for detection of microsporidia in their hosts, including immunological (Irby et al., 1986; Lujan et al., 1998; Oien and Ragsdale, 1992), Southern hybridization (Leiro et al., 1999;

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Malone and McIvor, 1995), and PCR amplification with species-specific primers (David et al., 1996; Leiro et al., 2002; Talal et al., 1998). Although each has its advantages, PCR is considered a superior method because it provides detection at very low levels of infection and can identify all stages of infection (Weiss and Vossbrinck, 1999). Here, we report the development of a multiplex PCR-based procedure for rapid, sensitive, and specific detection of *T. solenopsae* in all developmental stages of *S. invicta*.

2. Materials and methods

2.1. Spore purification

Spores of *T. solenopsae* were purified by isopycnic centrifugation on Redigrade colloidal silica (Undeen and Alger, 1971). *T. solenopsae*-infected colonies of polygynous *S. invicta* were excavated from areas in Gainesville, FL, and transferred to rearing trays using the floating technique described previously (Jouvenaz et al., 1977). Colonies were immediately assessed by phase-contrast microscopy at 400× to determine whether or not they were infected with *T. solenopsae*. Worker ants (~50 g) from infected colonies were blended on high speed for 30 s in spore buffer (50 mM Tris buffer, pH 7.3). The mixture was filtered through course glass wool and the filtrate centrifuged at 800*g* for 15 min at 4 °C. The supernatant was discarded, the pellets resuspended in spore buffer, and further centrifuged in a Beckman ultracentrifuge at 105,000*g* for 15 min at 4 °C. Pellets were resuspended in 3 ml of spore buffer and applied to a Redigrade:NaCl mixture (9:1; 0.15 M NaCl). The Redigrade mixture was centrifuged at 105,000*g* for 15 min at 4 °C. The *T. solenopsae*-containing band, corresponding to a density of approximately 1.13 g/ml, was removed by suction and isopycnic centrifugation was repeated with fresh Redigrade/NaCl. The spore-containing bands were again removed, diluted with spore buffer to 250 ml, and centrifuged at 500*g* for 15 min. The pellets were resuspended in spore buffer (1 ml) and the spore concentration determined on a hemacytometer. Spores were used immediately and all procedures were conducted at 4 °C.

2.2. Nucleic acid preparation

DNA preparations were identical for all ant stages and microsporidian species. Worker ants (20–100), larvae (5–50), eggs (100–500), pupae (5–25), prepupae (5–25) queens (1) or spores (10–10^6) were placed into a 1.5 ml microcentrifuge tube containing 150 μl of lysis buffer (50 mM Tris–HCl, pH 8, 4% sodium dodecyl sulfate, and 5% 2-mercaptoethanol). The insects were homogenized with a disposable plastic pestle for 15 s and the mixture was incubated at 100 °C for 15 min. When only spores were used, the homogenization step was omitted. The mixture was allowed to cool on ice for 1 min, followed by the addition of 200 μl of phenol:chloroform:isoamyl alcohol (Tris–HCl-saturated, pH 8). The mixture was inverted five times and centrifuged at room temperature for 5 min at 16,000*g*. The supernatant was removed and nucleic acids precipitated with ice-cold isopropanol and the pellets were washed twice with 70% ethanol. Pellets were dried at 37 °C, then dissolved in 30 μl of TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8).

2.3. Polymerase chain reaction

PCR was carried out using primer pairs we designed (Table 1) for the small subunit (SSU) rRNA gene of *T. solenopsae* (Moser et al., 1998; Moser et al., 2000; Accession No.: AF031538) and the *Gp-9* gene of *S. invicta* (Krieger and Ross, 2002; Accession No.: AF459414). The *T. solenopsae* SSUrRNA-specific primers corresponded to positions 164–182 (1TsS) and 457–481 (2TsAS) which produced a 318 bp amplicon. The *T. solenopsae*-specific primers were designed by aligning all available microsporidia SSUrRNA genes from GenBank with the Vector NTI 7.1 program (Informax, Bethesda, MD) and choosing areas unique to *T. solenopsae*. The *S. invicta Gp-9*-specific primers corresponded to positions 289–313 (7GP9S) and 758–782 (8GP9AS) which produced a 494 bp amplicon. In addition, a pair of universal primers was designed toward highly conserved areas of microsporidian SSUrRNA intended to amplify most microsporidia species (Table 1, Fig. 1). Primers 9UnivS and 10UnivAS corresponded to positions 869–890 and 956–974 of the *T. solenopsae* SSUrRNA gene, respec-

Table 1
List of oligonucleotide primers used in PCR

Name	Strand orientation	Sequence
1TsS	Sense	5′CGAAGCATGAAAGCGGAGC
2TsAS	Antisense	5′CAGCATGTATATGCACTACTGGAGC
7GP9S	Sense	5′TAAAATTCCAAATCTAGGCTTTCGC
8GP9AS	Antisense	5′CAAACATGAGAGTGCAGTGTGAACA
9UnivS	Sense	5′GCTTAATTTGACTCAACGCGGG
10UnivAS	Antisense	5′GAAAACGGCCATGCACCAC

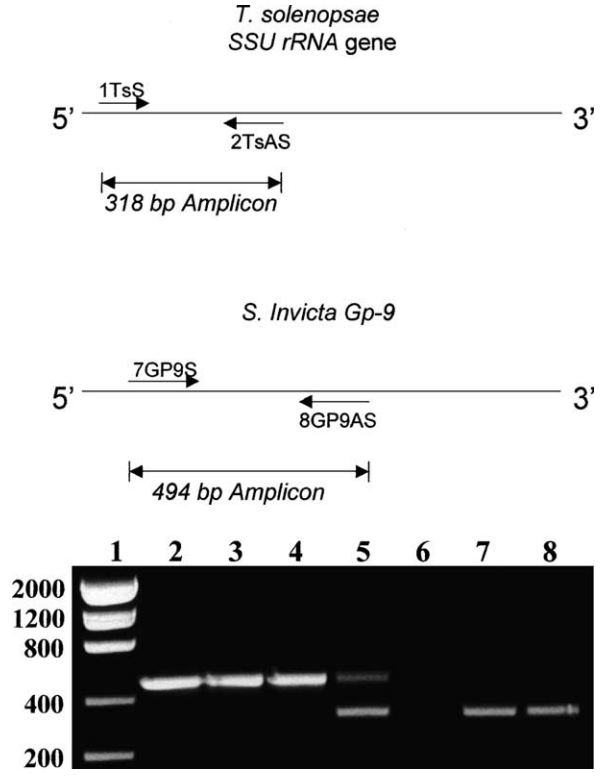

Fig. 1. Schematic representation of multiplex PCR to detect *T. solenopsae* in *S. invicta* and the banding patterns after separation on a 1% agarose gel for different ant/spore/primer combinations. *T. solenopsae* SSUrRNA gene is 1371 bp and the *Gp-9* gene is 2200 bp. Lane 1, molecular weight markers (bp); lane 2, uninfected *S. invicta* workers (primer pair 7GP9S, 8GP9AS); lane 3, uninfected *S. invicta* workers (primer pairs 1TsS, 2TsAS and 7GP9S, 8GP9AS); lane 4, *T. solenopsae*-infected *S. invicta* workers (primer pair 7GP9S, 8GP9AS); lane 5, *T. solenopsae*-infected *S. invicta* workers (primer pairs 1TsS, 2TsAS and 7GP9S, 8GP9AS); lane 6, *T. solenopsae* spores (primer pair 7GP9S, 8GP9AS); lane 7, *T. solenopsae* spores (primer pairs 1TsS, 2TsAS and 7GP9S, 8GP9AS); lane 8, *T. solenopsae* spores (primer pair 1TsS, 2TsAS).

tively. The universal primers were always used alone (i.e., not multiplexed) and generated an amplicon of approximately 115 bp depending on the species (e.g., *T. solenopsae* produced a 106 bp amplicon).

Multiplex PCR and single-primer pair PCR were conducted by the hot start method in a PTC 100 thermal cycler (MJ Research, Waltham, MA) under the following optimized temperature regime: 1 cycle at 94 °C for 2 min, then 35 cycles at 94 °C for 15 s, 55 °C for 30 s, and 68 °C for 30 s, followed by a final elongation step of 5 min at 68 °C. The reaction was conducted in a 50 μl volume containing 2 mM $MgCl_2$, 200 μM dNTP mix, 1 U Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), 0.4 μM of each primer, and 1 μl of the genomic DNA preparation (50–500 ng). PCR products (15 μl unless otherwise noted) were separated on a 1% agarose gel and visualized by ethidium bromide staining. For all experiments, positive and negative controls were run alongside treatments.

Purified spore and infected insect samples were analyzed by multiplex PCR (*T. solenopsae*-specific and *S. invicta Gp-9* primer pairs) and single-primer PCR (microsporidian universal primer pair). A number of different microsporidian species (provided by Drs. L. Solter, University of Illinois and J. Becnel, USDA-ARS) were evaluated, including, *Octosporea muscaedomesticae* (host Sarcophagidae), *Vairimorpha necatrix* (host *Pseudaletia unipuncta*), *Endoreticulatus* sp. (host *Hyphantria cunea*), *Nosema* sp. near *Nosema bombycis* (host *Malacosoma americanum*), *Vavraia culicis* (host *Helicoverpa zea*), *Brachiola algerae* (host *Helicoverpa zea*), *Vairimorpha invictae* (host *S. invicta*), and *T. solenopsae* (hosts *S. invicta* and *richteri*). Additional protozoan parasites (kindly provided by Dr. R. Pereira, USDA-ARS) were also evaluated, including, *Mattesia geminata* (host *Solenopsis geminata*), and *Mattesia* sp. (host *S. invicta*). DNA preparation was conducted using the method described above. Additionally, *Gp-9*-specific primers were evaluated against DNA preparations from *S. richteri*, *Solenopsis xyloni*, *S. geminata*, *S. invicta*/*richteri* hybrids and monogyne and polygyne *S. invicta*.

Two experiments were conducted to evaluate the detection limits of the multiplex PCR method. In the first experiment, 20–100 worker ants from a colony known to be uninfected were added to a microcentrifuge tube containing a single infected worker ant (verified by microscopic examination). DNA preparation and multiplex PCR were subsequently conducted as described. No attempt was made to determine the titer of spores in each of the infected ants. In the second experiment, we added a known quantity of spores (50–50,000 from purified preparations and preparations taken directly from infected ants) to a group of uninfected worker ants (20), purified the DNA, and conducted multiplex PCR. Each experiment was conducted three times.

We also examined each developmental stage of an infected colony for the presence of *T. solenopsae* using the multiplex PCR method. Eggs, 1st, 2nd, 3rd, and 4th instars, prepupae, pupae, adult workers, and queens were taken from an infected colony and analyzed by multiplex PCR.

3. Results

Fig. 1 illustrates the multiplex PCR method and the specificity of each primer pair for *S. invicta* and *T. solenopsae*. When used alone against DNA from *T. solenopsae*-infected and uninfected *S. invicta* workers (polygyne or monogyne), the 7GP9S and 8GP9AS (fire ant *Gp-9*-specific) primer pair produced a single 494 bp amplicon. Similarly, DNA from uninfected *S. invicta* workers produced a single 494 bp amplicon when 1TsS, 2TsAS (*T. solenopsae* SSUrRNA-specific), and 7GP9S,

Table 2
PCR results with various *Solenopsis* and protozoan species

DNA preparation	PCR amplification with primer pair[a]		
	1TsS/2TsAS	7GP9S/8GP9AS	9UnivS/10UnivAS
Formicidae			
S. invicta (monogyne)	–	+	–
S. invicta (polygyne)	–	+	–
S. invicta (polygyne *T. solenopsae*-infected)	+	+	+
S. richteri	–	+	–
S. invicta/richteri hybrid	–	+	–
S. geminata	–	+	–
Microsporidia			
Thelohania solenopsae	+	–	+
Octosporea muscaedomesticae	–	–	+
Vairimorpha necatrix	–	–	+
Vavraia culicis	–	–	+
Brachiola algerae	–	–	+
Vairimorpha invictae	–	–	+
Nosema sp.	–	–	+
Endoreticulatas sp.	–	–	+
Neogregarinae			
Mattesia geminata	–	–	+
Mattesia sp.	–	–	+

[a] "+" indicates amplification and "–" indicates no amplification with the designated primer pair.

8GP9AS primer pairs were used simultaneously (multiplexed). No amplification occurred with primer pair 7GP9S and 8GP9AS against DNA preparations from purified *T. solenopsae* spores. DNA preparations from purified *T. solenopsae* spores produced a single 318 bp amplicon in the presence of primer pairs 1TsS, 2TsAS, and 7GP9S, 8GP9AS. Similarly, DNA prepared from *T. solenopsae*-infected *S. invicta* workers produced a single 318 bp amplicon when the primer pair 1TsS, 2TsAS was used. When both primer sets (1TsS, 2TsAS, and 7GP9S, 8GP9AS) were used with DNA prepared from *T. solenopsae*-infected *S. invicta* (or uninfected *S. invicta* DNA amended with *T. solenopsae* DNA), 494 and 318 bp amplicons were produced.

A number of different species of *Solenopsis*, microsporidia, and neogregarinae were tested by PCR with different primer pairs to determine primer specificity (Table 2). The 1TsS and 2TsAS primer pair only amplified DNA from *T. solenopsae* and *T. solenopsae*-infected *S. invicta* and *S. richteri*. The *Gp-9*-specific primers (7GP9S and 8GP9AS) amplified all *Solenopsis* species evaluated, including *S. richteri*, *S. xyloni*, *S. geminata* the *S. invicta/richteri* hybrid, and monogyne and polygyne *S. invicta*. The microsporidian universal primer pair (9UnivS, 10UnivAS) amplified DNA from all of the microsporidia species tested, *T. solenopsae*-infected *S. invicta*, as well as the two *Mattesia* species.

The multiplex PCR method (primer pairs 1TsS, 2TsAS, and 7GP9S, 8GP9AS used simultaneously) detected *T. solenopsae* in all developmental stages of *S. invicta* (Fig. 2). Amplification of DNA derived from

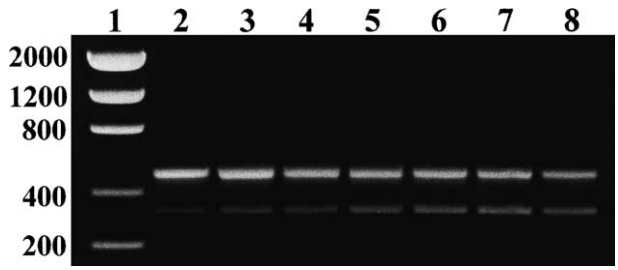

Fig. 2. Ethidium bromide stained 1% agarose gel electrophoresis of multiplexed PCR-amplified products of SSUrRNA and *Gp-9* of different developmental stages of *T. solenopsae*-infected *S. invicta*. Lane 1, molecular weight markers (bp); lane 2, eggs (30 μl); lane 3, 1st instars; lane 4, 2nd and 3rd instars; lane 5, 4th instars; lane 6, prepupae; lane 7, pupae; lane 8, queens.

eggs produced a weak signal despite high infection rates in the queens that laid them. *S. invicta* workers (20) amended with as few as 5000 spores were capable of being identified as positively infected by the multiplex PCR method (Fig. 3). However, when this experiment was repeated using only the *T. solenopsae*-specific primer pair, as few as 10 spores were detectable. Finally, a single *T. solenopsae*-infected worker ant was able to be identified as positively infected among at least 100 uninfected workers.

4. Discussion

Among the molecular techniques, PCR, restriction mapping, and hybridization probes, PCR has been

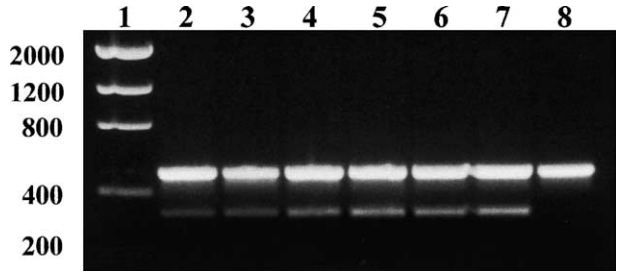

Fig. 3. Limits of detection of multiplex PCR for *T. solenopsae* infection of *S. invicta* worker ants. A known quantity of spores (5000–50,000) were added to 20 *S. invicta* worker ants in a microcentrifuge tube. DNA was extracted as described and multiplex PCR conducted. Lane 1, molecular weight markers (bp); lanes 2–7, 20 worker ants amended with 5000; 10,000; 20,000; 30,000; 40,000; and 50,000 spores, respectively; lane 8, uninfected ants.

most widely employed for microsporidian diagnosis and epidemiologic studies (see Weiss and Vossbrinck, 1999 for numerous examples). The SSUrRNA (16S) gene sequences of many microsporidia have been elucidated and found to diverge greatly from other eukaryotes; the sequence is shorter and shares little homology with other eukaryotes (Weiss and Vossbrinck, 1999). Thus, the SSUrRNA genes of the microsporidia possess characteristics amenable to molecular detection.

Currently, detection of *T. solenopsae* in its host, *S. invicta*, is limited to microscopic examination which is time consuming and requires considerable expertise, particularly for early stages of development (Knell et al., 1977). Indeed, the complete life cycle of *T. solenopsae* has not been elucidated. The *T. solenopsae* SSUrRNA gene-specific primers we developed, successfully amplified a 318 bp fragment from DNA preparations from purified *T. solenopsae* spores and all developmental stages of *T. solenopsae*-infected *S. invicta* (Fig. 2). Conversely, this primer pair did not amplify DNA preparations from uninfected *S. invicta*, seven additional species of microsporidia (including *V. invictae*), or *Mattesia* spp. (Table 2). Therefore, the primer pair, 1TsS and 2TsAS, exhibits specificity toward *T. solenopsae*.

The primer pair 7GP9S and 8GP9AS, specific for the *Gp-9* gene of *S. invicta* (Krieger and Ross, 2002), served as a positive control in multiplex PCR with the *T. solenopsae*-specific primer pair. Because *S. invicta* DNA is co-purified when examining ant samples for *T. solenopsae* infection by PCR, amplification of a portion of *S. invicta Gp-9* provides verification of successful DNA preparation. Consider a situation in which no amplification occurred in a sample using only the *T. solenopsae*-specific primer pair (1TsS and 2TsAS) for PCR. In this case, one would conclude that the ant sample was negative for *T. solenopsae* infection. However, a similar result (no amplification) would also occur if the DNA preparation failed. Therefore, multiplexing the *T. solenopsae*-specific primers with the *Gp-9* primers prevents false negative conclusions resulting from failed DNA preparations.

The universal primer pair (9UnivS, 10UnivAS) designed toward highly conserved areas within microsporidia SSUrRNA genes successfully resulted in amplification of DNA preparations from *T. solenopsae* (106 bp), seven additional microsporidia (~110 bp), and two *Mattesia* species (Table 2). Unfortunately, attempts to multiplex the universal primer pair with the other primer pairs (1TsS, 2TsAS and 7GP9S, 8GP9AS) resulted in inconsistent amplification of *T. solenopsae* SSUrRNA.

Several experiments were conducted to establish the level of detection of *T. solenopsae* in a sample of *S. invicta* worker ants. When 20 uninfected workers were amended with varying numbers of spores, amplification of the SSUrRNA gene occurred consistently to a lower limit of 5000 spores (Fig. 3). However, using only the *T. solenopsae*-specific primers, as few as 10 spores were detectable. Although the limit of detection for multiplex PCR was considerably lower than single primer PCR, it offered the advantage of a positive control conducted simultaneously. Moreover, a typical infected worker fire ant contains 3×10^5 to 3×10^6 spores (Moser, 1995) which is 6- to 60-fold higher than the multiplex PCR limit of detection. Presumably, the decreased resolution with multiplexing is caused by primer competition and an extreme abundance of *S. invicta* DNA compared with *T. solenopsae* DNA. The limit of detection of *T. solenopsae* by the single primer pair method (10 spores) was similar to PCR-based microsporidian detection methods reported previously (Katzwinkel-Wladarsch et al., 1996; Leiro et al., 2002).

We also added a single infected individual worker to increasing numbers of uninfected worker ants (20–100) to identify any limitations imposed by sample size. *T. solenopsae* SSUrRNA gene amplification successfully occurred at a ratio as high as 1:100 (infected to uninfected worker ants). We did not examine larger numbers of worker ants (higher ratios) because microcentrifuge tube limitations occurred beyond 100 ants. Therefore, the method, as described, was capable of detecting a single infected worker ant among 100 uninfected ants. These data demonstrate the sensitivity of PCR for *T. solenopsae* detection.

The multiplex PCR method to detect *T. solenopsae* infection in *S. invicta* offers significant advantages over traditional microscopic techniques, including, an increase in the level of detection sensitivity (Leiro et al., 2002), lower risk of misidentification, and the ability to detect the infection in all stages of fire ant. Further, the multiplex PCR reaction will facilitate epidemiological studies by permitting rapid identification of *T. solenopsae* and provide a means of more easily screening potential intermediate host organisms.

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